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Comparison of Polyphenol Oxidase Expression in Glandular Trichomes of Solanum and Lycopersicon Species

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ABSTRACT

Tetrlobulate glandular trichomes are present on the foliage of many solanaceous species. Resistance of many of these species to insects is conditioned by the ability of trichomes to rupture upon contact and to rapidly polymerize their contents, resulting in entrapment of insects in hardened trichome exudate. In the wild potato, Solanum berthaultii, polymerization of trichome exudate is initiated by a soluble M, 59,000 polyphenol oxidase (PPO), which is a dominant protein constituent of the organ. PPOs, although ubiquitous in angiosperms, typically display great heterogeneity in molecular weight and are found at low levels in plant cells. Because of the unusually high accumulation and tissue-specific expression of the M, 59,000 PPO in S. berthaultii glandular trichomes, we analyzed trichome proteins of a number of Lycopersicon and Solanum species to assess the extent to which possession of the M, 59,000 PPO is conserved. Trichomes were collected manually and examined for PPO activity, immuno-cross-reactivity with S. berthaultii M, 59,000 PPO, and protein content. In addition, N-terminal amino acid sequences were obtained for five trichome PPOs. All species analyzed possessed trichome PPOs similar in structure and level of expression to that of S. berthaultii. The relationship between sequences and structures of these conserved PPOs and the variable PPOs of leaf is discussed.

PPOs (EC 1.10.3.2, 1.14.18.1) are copper metalloproteins that catalyze the oxidation of phenols to quinones via the reduction of O2. PPOs are ubiquitous among angiosperms (21) and appear to be localized in plastids in both photosynthetic and nonphotosynthetic cells of many organs and tissues, where they are present at low levels. Although a large number of biochemical studies have been conducted since PPO was first described in 1895, the physiological function(s) of PPO remain obscure (16–18, 25, 26). PPOs from green leaves are among the most well studied of these enzymes and are recognized primarily as plastidic copper metallopro-

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4 Abbreviations: PPO, polyphenol oxidase; IEF, isoelectric focusing; PI, isoelectric point.

MATERIALS AND METHODS

Plant Materials

Plants of Solanum berthaultii Hawkes, plant introduction number (PI) 473334, S. tarajense, S. neocardenosii, S. polyadenium, S. tuberosum ssp. andigena, Lycopersicon esculentum (Xa-872086-2), cv VFNT, cv Freedom, and cv New Yorker, L. chmielewskii (LA 1316), L. cheesmanii (LA 483), L. peruvianum (LA 462), L. hirsutum (LA 1736), L. pennelli (LA 1272), L. parviflorum PI (LA 1321), and L. pimpinellifolium (LA 1061)
were grown in the greenhouse under 1000-W metal halide lamps with a 16-h photoperiod.

Glandular Trichome Collection
Type A glandular trichomes (24) (also known as type VI in *Lycopersicon* [14]) are 120 to 210 μm in length, each with a 50- to 70-μm tetrabulbate head (composed of four to eight cells) at its apex. For each electrophoretic analysis, type A (*Solanum*) or type VI (*Lycopersicon*) trichomes were manually collected by touching each trichome head with the end of a 300-μm diameter glass capillary that had been drawn out to a diameter of approximately 50 μm. Upon contact, the trichomes rupture and their contents are drawn into the capillary, which is filled with 200 mM DTT. For silver staining, gels were loaded with the contents of 400 trichomes. For western analysis with polyclonal anti-trichome PPO antibody, 50 trichomes were collected, electrophoresed, transferred, and developed with anti-*S. berthaultii* trichome PPO (12). For PPO activity staining, the high numbers of individual trichomes required to detect activity made it necessary to obtain trichome PPOs by wiping leaflets. Therefore, 30 young leaflets, representing approximately equal leaf areas for each species, were wiped abaxially and adaxially with cotton swabs saturated with 150 mM DTT. Crude fractions were squeezed from the swab with a syringe and centrifuged at 13,000g for 15 min at 4°C. Twenty microliters of the supernatant was mixed with pH 3.0 to 10.0 ampholytes (Bio-Rad) to a concentration of 2% and loaded onto IEF gels, which were developed for PPO activity as described below. Samples for activity staining were compared on the basis of equal leaflet area rather than on the basis of equal protein. Mechanical damage to epidermal and subepidermal tissues frequently occurs when wiping leaves to sample trichome contents, and the severity of damage is related to the toughness of different species' leaflets. As a result, nontrichomal proteins are sometimes represented in leaf wipe extracts, rendering comparisons of PPO activity on the basis of equal protein invalid.

Electrophoresis, Activity, and Immunostaining
SDS-PAGE was performed on a 10% polyacrylamide minigel system (Bio-Rad Mini-Protein II). IEF was performed on 5% polyacrylamide minigels with a pH gradient of 3 to 10 (19). IEF gels were developed with silver stain (11) or were stained for PPO (catechol oxidase) activity after electrophoresis by placing the gels in a solution of 0.1 M sodium phosphate, pH 7.0, 0.3 M catechol, and 90 mM p-phenylene-diamine for 5 to 10 min. The gels were then thoroughly washed with water followed by a 2-min rinse in 10% (w/v) citric acid. Gels were then dried and stored at 20°C.

Before transblotting, SDS-PAGE and IEF gels were equilibrated for 20 min in a transfer buffer of 25 mM Tris, 192 mM glycine, and 20% (v/v) methanol, pH 8.3, and then transferred to nitrocellulose at 0°C, 100 V, 0.25 A, for 1 h in a mini-transblot unit (Bio-Rad). Development of immunoblots was done according to the procedure described by Ausubel et al. (1) using a 1:3000 dilution of polyclonal anti-*S. berthaultii* M, 59,000 PPO that had been purified by ammonium sulfate fractionation and reconstituted in PBS to its original concentration in the antiserum (11, 12). Goat-anti-rabbit immunoglobulin G/alkaline phosphatase conjugate (Bio-Rad) was used as a second antibody according to the manufacturer's instructions.

Analysis of Trichome PPO Amino Acid Composition and N-Terminal Amino Acid Sequence
Approximately 600 leaflets were wiped abaxially and adaxially with 20 swabs each saturated with ice-cold 200 mM DTT. The crude trichome extract was immediately squeezed out of the cotton swab with a syringe and mixed with one-tenth volume of ice-cold 100% TCA and centrifuged at 13,000g for 5 min. The pellet was washed twice with 80% acetone and centrifuged at 13,000g for 5 min. After acetone was removed in vacuo, the pellet was resuspended in 200 μL of SDS loading buffer, boiled for 3 min, and electrophoresed in 10% SDS-PAGE gels. Proteins were transferred from the gels to Immobilon-P polyvinylidene difluoride membranes in 10 mM Caps, 10% methanol buffer (pH 11) by electroblotting at 0.5 A of constant current for 25 min. The membranes were developed with Coomassie blue R-250 and destained according to the method of Matsudaira (15). The bands corresponding to trichome PPO were excised and submitted to the Cornell University Biotechnology Center for amino-terminal microsequencing by automated Edman degradation and for amino acid composition analysis after hydrolysis and derivatization (Pico-Tag, Waters Associates).

RESULTS

Protein Profiles of Trichomes
Four hundred type A or type VI trichomes were collected manually, electrophoresed on IEF gels, and developed for protein by silver staining. Type A and type VI trichomes of all 13 species possessed protein profiles that were dominated by a single band constituting the bulk of soluble trichome protein. In all cases, this major protein band focuses at a pH between 5.3 and 5.5 (Fig. 1). The major trichome protein from the five *Solanum* species surveyed (data not shown) focuses at pH 5.5, the pl of *S. berthaultii* trichome PPO (Fig. 1). In contrast, the major trichome protein from the eight *Lycopersicon* species possesses a pl of about 5.3 (Figs. 1–3).

PPO Activity
For activity staining trichomes were collected and their contents were separated by IEF and exposed to catechol. PPO activity was observed in trichome preparations from all species (Fig. 2). Although the intensity of staining varied, in all cases activity was observed at a pH between 5.5 and 5.3, similar to the pl of the dominant trichome protein and similar to the pl previously reported for the M, 59,000 trichome PPO of *S. berthaultii*. 
Immuo-Cross-Reactivity of PPO in Trichomes

Fifty trichomes were manually collected and electrophoresed on IEF gels; the proteins were transblotted and probed with antibodies to the *S. berthaultii* M, 59,000 trichome PPO. The results (Fig. 3) indicate that trichomes from the species examined possess a single protein constituent that strongly cross-reacts with the trichome PPO antibodies. In all species, this band corresponds to the most abundant protein of the trichome and to the band detected by enzyme staining.

The order of signal intensities for the immunological, protein, and PPO activity stains for the trichome PPOs is in general agreement. However, the PPO activity staining for *L. peruvianum* is relatively weak compared with its strong immunostaining with anti-PPO. The weak activity stain exhibited by *L. peruvianum* trichome PPO may be explained by low activity of this enzyme toward catechol. Alternatively, the weak PPO activity may reflect the low density of type VI trichomes on *L. peruvianum* leaflets, which affects the amount of PPO harvested from trichomes by leaf wipes. In contrast, the immunological and protein analyses are not affected by trichome density because trichomes are collected manually. *L. hirsutum* also shows a much weaker staining for PPO activity than is suggested by the intensity of its immunostaining. This difference suggests that *L. hirsutum* trichome PPO may be weakly active toward catechol, because the density of type VI trichomes on this species is quite high. Although it is possible that the *L. hirsutum* and *L. peruvianum* trichome PPOs become partially inactivated during the isolation procedure or possess high activity with substrates other than catechol, we did not explore these possibilities.

Amino Acid Composition and N-Terminal Amino Acid Sequences of Trichome PPOs

All species analyzed possess trichome PPOs with *M* of approximately 59,000 (Table I). PPOs from *S. berthaultii*, *L. cheesmanii*, and two cultivars of *L. esculentum* were transferred from SDS-PAGE to Immobilon filters and subjected to amino acid composition analysis and N-terminal microsequencing. After 14 to 19 cycles of automated Edman microsequencing, the N-termini of trichome PPOs showed a high degree of similarity (Table I). Deviations from the observed homology are minimal: the first residue in *S. berthaultii* trichome PPO is Ser instead of Ala. The identity of residue 11 was ambiguous for all species examined. It is unclear whether this result is due to heterogeneity of the preparation (e.g., expression of a family of PPOs) or to covalent modification of this residue by quinones enzymically generated by PPO. Residue 11 is Cys in the predicted N termini of a VFNT cultivar tomato trichome PPO cDNA clone (27) and a PPO cloned from tomato floral tissue (20).

The N-terminal sequences of the trichome PPOs were also compared with those of four broad bean (*Vicia faba*) PPOs determined by Flurkey (6) (Table I). The N-terminal sequences of the four broad bean PPOs and the five trichome PPOs have seven identical and three isoformal amino acids out of 12 residues. Residues 9 and 10 (Lys-Ser) in the trichome PPOs are inverted relative to broad bean PPO. Similar to the tomato sequence, the identity of residue 11 was difficult to assign for two of the four *Vicia* PPO species (6).
The amino acid compositions of these PPOs are shown in Table II. Glu and Gln are grouped as Glx, and Asp and Asn are grouped as Asx because they could not be distinguished by the analytical method used. The contribution of Trp residues was also not determined because Trp is destroyed during acid hydrolysis. The proportion of each amino acid residue is highly conserved, with differences among these species ranging from 0.4 to 3.5% for each amino acid residue (Table II). The greatest difference observed was for Pro: 3.4% between PPOs from *L. esculentum* cv Freedom and *L. cheesmanii*. The smallest difference observed was for Thr (0.4%). For the majority of amino acid residues, the difference among individual residues is low, in agreement with the biochemical similarities of the PPOs determined above. Although there is a distinct difference between the pI of *Solanum* (pl 5.5) and *Lycopersicon* (pl 5.3) PPOs, this difference is not reflected in their composition of basic residues and may reside instead in a greater proportion of Asp and Glu residues in *Lycopersicon* PPOs.

### DISCUSSION

This survey of trichome PPOs from *Solanum* and *Lycopersicon* species demonstrates that PPO is present as the dominant protein in the tetralobulate glandular trichomes of many *Solanum* and *Lycopersicon* species. These glandular trichome PPOs exhibit substantial conservation of primary amino acid sequence, amino acid composition, M₉, pl, and immuno-cross-reactivity.

We previously calculated that approximately 1 ng of PPO is present in a single *S. berthaultii* type A trichome (or a concentration of 14 mg/mL, assuming no compartmentalization) (9, 11). This study shows that the high level of PPO expression exhibited by *S. berthaultii* is shared by other Solanaceae. These similarities suggest that the M₉, 59,000

### Table I. Comparison of N-Terminal Amino Acid Sequence of *S. berthaultii* Trichome PPO with Trichome PPOs from Four *Lycopersicon* Species and Leaf PPOs from *V. laba*

<table>
<thead>
<tr>
<th>Species</th>
<th>M₉</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>L. esculentum</em> (cv VFNT)</td>
<td>58,600</td>
<td>A-I-P-I-P-P-D-L-K-S-X-G-T-A</td>
</tr>
<tr>
<td><em>L. esculentum</em> (cv Freedom)</td>
<td>58,500</td>
<td>A-I-P-I-P-P-D-L-K-S-X-G-T-A</td>
</tr>
<tr>
<td><em>V. laba</em> (A1)</td>
<td>61,500</td>
<td>S-I-P-I-S-P-D-L-K-S-K-X-G-P-P</td>
</tr>
<tr>
<td><em>V. laba</em> (A2)</td>
<td>60,000</td>
<td>S-I-P-I-S-P-D-L-S-X-I-G-P-P</td>
</tr>
<tr>
<td><em>V. laba</em> (B1)</td>
<td>44,500</td>
<td>S-I-P-S-P-D-L-K-X-G</td>
</tr>
<tr>
<td><em>V. laba</em> (B2)</td>
<td>43,000</td>
<td>S-I-P-S-P-D-L-K-N-G</td>
</tr>
</tbody>
</table>

* Vicia PPO sequence information is from Flurkey (6).  

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### Table II. Comparison of Amino Acid Composition among Trichome PPOs

<table>
<thead>
<tr>
<th>Species</th>
<th>Asx</th>
<th>Glx</th>
<th>Ser</th>
<th>Gly</th>
<th>His</th>
<th>Arg</th>
<th>Thr</th>
<th>Ala</th>
<th>Pro</th>
<th>Tyr</th>
<th>Val</th>
<th>Met</th>
<th>Cys</th>
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<th>Leu</th>
<th>Phe</th>
<th>Lys</th>
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</thead>
<tbody>
<tr>
<td><em>S. berthaultii</em></td>
<td>11.0</td>
<td>9.6</td>
<td>5.2</td>
<td>8.8</td>
<td>2.6</td>
<td>4.4</td>
<td>5.2</td>
<td>5.6</td>
<td>7.4</td>
<td>7.4</td>
<td>6.3</td>
<td>1.6</td>
<td>2.3</td>
<td>5.2</td>
<td>6.9</td>
<td>5.8</td>
<td></td>
</tr>
<tr>
<td><em>L. chmielewskii</em></td>
<td>14.2</td>
<td>10.2</td>
<td>6.9</td>
<td>7.5</td>
<td>2.2</td>
<td>5.0</td>
<td>5.6</td>
<td>6.4</td>
<td>7.2</td>
<td>4.0</td>
<td>5.7</td>
<td>1.8</td>
<td>4.3</td>
<td>5.2</td>
<td>7.5</td>
<td>6.0</td>
<td></td>
</tr>
<tr>
<td><em>L. cheesmanii</em></td>
<td>13.2</td>
<td>9.4</td>
<td>7.0</td>
<td>9.3</td>
<td>1.4</td>
<td>5.0</td>
<td>5.2</td>
<td>7.0</td>
<td>6.4</td>
<td>4.2</td>
<td>6.4</td>
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<td>5.2</td>
<td>8.1</td>
<td>6.2</td>
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</tr>
<tr>
<td><em>L. esculentum</em> (cv VFNT)</td>
<td>12.6</td>
<td>10.0</td>
<td>6.8</td>
<td>6.8</td>
<td>1.8</td>
<td>4.8</td>
<td>5.5</td>
<td>6.0</td>
<td>8.7</td>
<td>4.0</td>
<td>5.9</td>
<td>0.9</td>
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<td>5.5</td>
<td>8.0</td>
<td>5.6</td>
<td></td>
</tr>
<tr>
<td><em>L. esculentum</em> (cv Freedom)</td>
<td>12.8</td>
<td>10.5</td>
<td>6.9</td>
<td>7.4</td>
<td>2.2</td>
<td>4.9</td>
<td>5.5</td>
<td>6.3</td>
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<td>7.4</td>
<td>3.0</td>
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</table>
trichome PPO evolved well before the diversification of the Solanaceae. The occurrence and the high accumulation of the Mr 59,000 PPO in the tetralobulate glandular trichomes is remarkable in terms of both tissue specialization and protein sequestration and illustrates the degree to which plants have evolved defenses against insect herbivores. These findings also suggest that the Mr 59,000 epidermal PPO promoter could serve as a vehicle to target the delivery of allelochemical related genes (or other genes involved in interspecies interactions) to epidermal cells of many higher plant species.

Although the Mr 59,000 trichome PPO and the nonepidermal PPOs appear to differ in Mr, substrate specificity, solubility, latency, tissue, and subcellular localization, level of expression, and (presumably) physiological function, the Mr 59,000 PPO antiserum cross-reacts with the nonepidermal PPOs (9). Despite the wide variability of Vicia PPO Mr, (43,000–61,000), the first 10 residues of all four Vicia polypeptides are identical. Furthermore, the mature N-terminal amino acid sequences of five Mr, 59,000 epidermal PPOs are highly similar to those of Vicia leaf PPOs (7 of 12 residues were identical, and 2 were inverted). Therefore, there appears to be a fairly close evolutionary relationship between these forms of PPO.

These findings have additional implications for understanding the targeting of PPOs to plastids and for the relationship between trichomal and nontrichomal PPOs. Both classes of PPOs are encoded by nuclear genes (10, 13). The trichome PPO is synthesized as an M, 67,000 precursor that accumulates in trichomal and epidermal leucoplasts as a soluble, Mr, 59,000 enzyme (27). Within the leucoplast, this PPO forms an amorphous protein body delimited by the thylakoid lumen. In contrast, the nonepidermal PPOs are membrane bound on the thylakoid, and cytochemical studies show that their enzymic reaction product accumulates on the luminal side of the membrane (21). The Mr, 40,000 to 45,000 nonepidermal PPOs, in contrast to the Mr, 59,000 trichome PPOs and most other nuclear-encoded thylakoid proteins (8), appear to be synthesized and incorporated into plastids without undergoing a detectable change in Mr, (4, 5, 22). Despite the apparent differences both in processing and in the subcellular localization of these two PPOs, our results show a remarkable degree of similarity between the mature N termini of these enzymes. The Mr, of trichome PPOs (59,000) and the Mr, 60,000 Vicia isoforms, taken together with the similarity in their N termini, suggest that the Mr, 60,000 Vicia leaf PPOs may undergo processing similar to that seen for the trichome PPOs. The possibility that the Mr, 40,000 to 45,000 Vicia PPOs may be derived posttranslationally from processed Mr, 59,000 to 60,000 PPOs (7) is supported by the N-terminal sequence similarity they share with the Mr, 59,000 to 60,000 proteins.

Recently, Dry and Robinson (2) provided evidence that the Vicia leaf PPO is a 60-kD protein that is susceptible to C-terminal proteolysis, leading to formation of an enzymically active 42-kD product. Clearly, the questions of both the possible artificial origin and the failure to note posttranslational processing of Mr, 40,000 to 45,000 leaf PPOs require further examination (7). Future analysis of PPO cDNAs should help to resolve differences in primary structure, processing, and organellar targeting of PPOs.

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LITERATURE CITED


